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IMMUNOGENIC PILI PRESENTING FOREIGN PEPTIDES, THEIR PRODUCTION AND USE

FIELD OF THE INVENTION

The present invention relates to pili presenting foreign peptides in the papA region, their production and use.

BACKGROUND OF THE INVENTION

U.S. Patent No. 4,740,585 discloses peptide vaccines for urinary tract infections prepared from synthetic peptides based on short sequences contained in HUR849 pilin A.

U.S. Patent No. 4,736,017 discloses peptide vaccines for urinary tract infections prepared from purified whole Gal-Gal pilus proteins or fragments thereof.

Baga et al., *Cell*, 49(1987): 241-251, disclose *papH* deletion mutants in one strain of *Escherichia coli*. The reference indicates that, in these *papH* deletion mutants, 50%-70% of total pilus antigen was found free of cells in the form of polymerized structures. Further, the dissociated and purified pili from these mutants are stated to agglutinate erythrocytes, though data is not disclosed. The reference does not indicate whether such dissociated pili from the *papH* mutant in polymerized form are effective as vaccines or whether these pili from the *papH* mutant would have altered antigenicity. In addition, the reference does not give the exact sequences of the two *papH* deletion mutants from the one strain of *E. coli*.

Van Die proposed (*J. Bacteriology*, 170: 5870-5876, 1988) using a gapped-duplex method with the F11 *pap* operon for insertion of a foreign peptide. However, his site of insertion did not correspond to the immunodominant site of papA.

SUMMARY OF THE INVENTION

An embodiment of the present invention is immunogenic pili presenting at least one foreign peptide in an immunodominant region of papA.

Another embodiment of the present invention is an immunogenic composition, including a vaccine against *E. coli* urinary tract infections or for other microbial infections/disease, where at least one epitope is expressed at the immunodominant region of PapA, comprising dissociated pili that are obtained after standard shearing methods from a Gal-Gal pilus-producing bacteria having at least one peptide inserted into the immunodominant epitope region of PapA that normally does not contain such a peptide sequence. The foreign epitope may be from another region of papA that is not immunodominant, or it may be entirely foreign to papA such as an HIV epitope.

Another embodiment of the present invention is a process for producing pili and vaccines comprising pili (including vaccines for urinary tract infections and other microbial infections/diseases) of the invention comprising culturing a recombinant Gal-Gal pilus-producing bacteria which expresses at least one foreign peptide at the immunodominant region of a PapA region that normally does not contain such a peptide, recovering dissociated pili, and formulating a vaccine comprising these pili. Preferably, these pilus-producing bacteria harbor at least one *papH* mutation that facilitates detachment of the pili from the bacteria relative to a wild type strain.

Another embodiment of the present invention is a method of treating or preventing a urinary tract infection or other microbial infection/disease disease where protective epitopes are expressed at the immunodominant region of PapA comprising administration to a subject in need thereof a vaccine produced according to the invention.

Another embodiment of the present invention are *E. coli* bacteria having novel mutations that result in hybrid pili that can express one or more foreign antigens in the immunodominant region of papA, which optionally further include a mutation that facilitates detachment of pili from the surface of bacteria relative to a wild type strain. Preferably, the mutation that facilitates detachment is a mutation in papH.

Another embodiment of the present invention is a plasmid that allows for the constant expression of immunologically novel pili at the surface of bacteria. This plasmid,

which preferably is a shuttle vector, could be used to transform live, attenuated microbes (e.g., aromatic mutant *Escherichia coli, Salmonella typhi, Salmonella typhimurium, Actinobacillus pleuropneumoniae)* as vaccine vehicles for small peptides (preferably spanning less than 20 amino acids). Also, simultaneous incorporation of the unique *papA* cassette and *papH* mutants provide for the constant release of hybrid Gal-Gal binding pili by transformed live attenuated microbes *in vivo* in animals and humans. Thus, the invention provides a method for either transient or chronic antigenic stimulation of a host with epitopes constituting protective immunity.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a genetic and physical map of recombinant plasmids used in the present *papH* invention.

Fig. 2(a) provides the *papH* DNA sequence of pHUR849, Fig. 2(b) pDAL201B *papH*, Fig. 2(c) pDAL210B *papH*, and Fig. 2(d) pDAL200A.

Fig. 3 provides a comparison of the *papH* DNA sequences of pHUR849, pDAL200A, pDAL201B, and pDAL210B.

Fig. 4 gives a comparison of deduced amino acide sequences of papH genes for pHUR849, pDAL200A, pDAL201B, and pDAL210B.

Fig. 5(a) shows the amino acids (which are underlined) that are deleted from papH in pHUR849 and Fig. 5(b) shows the amino acids (which are underlined) that are deleted from papH in pDAL201B, pDAL210B, and pDAL200A.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

An embodiment of the present invention is an immunogenic composition, including a vaccine for urinary tract infections, comprising dissociated pili from a recombinant pilus-producing bacteria, said pili comprising at least one immunogenic peptide inserted into a PapA region that does not normally contain such a peptide in the corresponding wild type. The inserted immunogenic peptide(s) may be a non-pilus-associated peptide or a pilus-associated peptide, including the substitution of PapA immunogenic epitopes located normally in other regions of the PapA. For example, cryptic

immunogenic PapA epitopes located at the amino terminal (corresponding to Residues 4 through 15) can be inserted into the immunodominant region and be rendered antigenic and immunogenic in the expressed hybrid Gal-Gal binding pili. Preferred immunogenic peptides to be inserted in the PapA region for the prevention of *E. coli* urinary tract infections are set out in the examples below. Preferably, the peptide is inserted into a position between amino acid residues 64 through 80 of the papA region. In another preferred embodiment, the foreign peptide is inserted by replacing 18 to 60 bases at the DNA level, which is a location within papA that corresponds to the immunodominant region of papA moiety and that corresponds to amino acid residues 65 through 75 in wild type papA.

Preferably, the pili of this embodiment are produced by culturing a bacteria having at least one mutation that facilitates detachment of the pili from the bacteria relative to a wild type strain. Preferably, the mutation is one that impairs or eliminates the anchoring function of papH.

Another embodiment of the present invention is a process for producing pili or a vaccine comprising pili for urinary tract infections comprising culturing a recombinant pilus-producing bacteria expressing pili comprising at least one immunogenic peptide inserted into a papA region that normally does not contain such a peptide and recovering detached pili from the culture. Optionally, the method further comprises formulating a vaccine comprising the pili for the prevention of *E. coli* or the treatment or prevention of other microbial infections/diseases if foreign protective epitopes are inserted into the immunodominant region of PapA. Preferably, detached pili are recovered from the culture by centrifugation and further purified by cycles of magnesium sulfate precipitation and tris solubilization. Still more preferably, the recombinant hybid PapA Gal-Gal pilus-producing bacteria further includes at least one mutation that facilitates detachment of the pili from the bacteria.

In one particular embodiment of the pili production method, a one-liter TSB culture yields about 10 mg of purified pili after 18 hours growth at 37 °C from each of the 4 recombinant strains that harbor *PapH* mutations.

Another embodiment of the present invention is a method of treating or preventing a urinary tract infection or other microbial infections/diseases (e.g., patients infected with HIV-1 or HIV-2 with or without AID-defining illness) if the protective epitope is inserted into the PapA immunodominant region comprising administering to a subject in need thereof a vaccine produced according to the invention.

Another embodiment of the present invention are *E. coli* bacteria having novel mutations that facilitate detachment of pili from the surface of bacteria by impairing or eliminating the anchoring function of PapH.

Another embodiment of the present invention relates to creation of novel protein-base immunogenic sequences set out in the examples below.

Preferably, the pilus-producing bacteria of the invention is *E. coli*, more preferably those disclosed in the examples below.

In the present invention, any mutation may be used that facilitates detachment of pili from the bacteria relative to a wild type strain. Preferably, the mutation impairs the anchoring function of PapH relative to a wild type pilus-producing bacteria, thereby increasing the amount of dissociated pili from the bacteria found in the culture supernatant relative to a wild type pilus-producing bacteria. Preferably, the mutation is a deletion mutation in the DNA encoding PapH, but other types of mutations achieving the same function may be used, such as insertion mutations.

Vaccines comprising the dissociated pili of the invention are formulated according to known methods, including those described in U.S. Patent No. 4,736,017. Suitable adjuvants may be used in the vaccines. The method of preventing *E. coli* urinary tract infections includes those described in U.S. Patent No. 4,736,017.

Adherence of *Escherichia coli* to uroepithelial cells is an important pathogenic step in the development of urinary tract infections. There are a number of adhesins expressed by uropathogenic *E. coli*, which may mediate uroepithelial attachment; however, pyelonephritogenic strains are characterized by the high frequency of pili associated with the α-D-Galp-(1-4)-β-D-Galp (Gal-Gal) binding. The Gal-Gal binding phenotype is considered critical to the pathogenesis of unobstructive, ascending urinary tract infection in anatomically normal, otherwise healthy young women. This is probably

due to the absence of a natural host defense factor in urine to prevent the Gal-Gal binding to uroepithelial cells. Digalactoside-binding adherence is mediated by pili, which are also known as Pap pili, or P pili because they bind to the P_1 blood group antigen (a globoside containing Gal-Gal) that is present on human erythrocytes and all epithelial cells.

The *pap* operon consists of at least 9 genes (1) that are required for the expression of the Pap pilus-adhesion complex (see Figure 1). PapA is the major (structural) fimbrial subunit. PapH is involved in both the termination of pilus growth and is required to anchor the fully grown pilus to the cell surface. PapC is located in the outer membrane and forms the assembly platform for pilus growth. PapD is a periplasmic which protein that forms complexes intracellularly with the pilus subunits before assembly. PapE, PapF, and PapG are tip pilus components. PapG is the adhesion molecule conferring Gal-Gal binding specificity. PapF complexes with PapG, and PapE attaches to PapA moieties, as well as, attaches and orients the PapF-PapG complex so that the adhesin is at the tip of the pilus.

The present invention is further illustrated by, though in no way limited to, the construction of hybrid PapA pili and likely protective epitopes against *E. coli* urinary tract infection to be inserted into the immunodominant region of PapA.

The immunodominant regions of F71, F72, F9, and F13 have been localized to the PapA moiety, specifically residing between residues 65-77, residues 65-77, residues 65-75, and residues 65-75, within the operon of pDal201B, pDal210B, pDal 200, and pHU849, respectively.

Furthermore, immunodominant fine deletion mutants in plasmids harboring the F operon for F71, F72, F9, and F13 (viz., pDal201B, pDal210B, pDal 200, and pHU849) have been performed. Linearized pDal201B, pDal210B, pDal 200, and pHU849 have been treated with endonuclease enzymes as recommended by the manufacturer. They were used to cleave the operon specifically at sites encompassing or within the immunodominant PapA region and without generation of too many or ambiguous fragments during digestion. The strategy included the following:

F type	Endonuclease restriction deletion	Deletion location at Pap
F71	*CviJI*	58-74
F72	*Aci*I	71-91
F9	*CviJI*	57-76
F13	*Mse*I	67-77

Oligonucleotide primers corresponding to the cleavage sites in proper orientation separated by desired sequences to be incorporated into the chimeric *papA* template are synthesized by conventional techniques. They are used to fill the cleaved recessed termini. A salmonella flagellin epitope corresponding to 6 amino acids and an epitope of human interleukin-4 peptide corresponding to 20 amino acids have been inserted into *papA* genetic cassettes. These genetic templates in *Escherichia coli* HB101 strain result in pili being expressed at the surface of the bacterium as demonstrated by electron microscopy and expression of hybrid pili as demonstrated by simultaneous binding of a single protein band in SDS-PAGE gels by Western blotting using polyclonal murine antibody to the pili and foreign epitopes. Cohorts of 5 mice each immunized with the hybrid product can elicit antibodies to the foreign epitope as demonstrated in ELISA tests.

The following peptide conjugate vaccines were demonstrated to be protective after serial parenteral administration in the BALB/c murine model of experimental pyelonephritis after intravesicular administration:

F serotype	Structural Pilin Sequence	Residue Position (R)
F13	PQGQGKVT	R 5-12
F13	AKFGGMGAKKG	R 65-65

Identification of additional protective epitopes of Pap pili and F1C structural pilin epitopes for the protection of pyelonephritis and cystitis, respectively, was accomplished. It should be noted the identified protective F11 epitope corresponding to residues 4 through 15 within the pilin A is essentially identical with F13 protective epitope in this region. However, a longer sequence was used for demonstrative purposes.

Peptides were synthesized by conventional solid phase techniques using tertiary butyloxycarbonyl-protected amino acids and amino acid polystyrene resins. As side chain protecting groups, O-benzyl esters were used for Asp, Glu, Thr and Ser, and tosyl groups were used for Arg and His. Cys was protected by ρ–methoxybenzyl, Lys by o-chorobenzyloxy-carbonyl, and Try by 2,6-dichlorobenzy. Couplings were performed with molar excess of τ–Boc amino acid and dicyclohexylcarbodiimide (DCC). If Asn or Gln was to be coupled, a molar excess of N–hydroxytriazole was included. Anydrous hydrogen fluoride in the presence of dimethylsulfide and anisol was used to cleave the protecting groups and the resin simultaneously. After either washing and/or acetic acid extraction, the purity of the final product was determined by reverse phase high performance liquid chromatography. The peptides were considered to be >98% pure following this conventional technique.

Conjugation of peptides to thyroglobulin or bovine serum albumin was performed using m-maleinimidobenzoyl *N*-hydroxysuccinimide ester (MBS) and succinimidyl 4-(*N*-maleinimido-methyl) cyclohexane-1-carboxylate (SMCC), respectively. The resulting peptide carrier conjugate was subsequently isolated by gel filtration. The molar ratio of conjugated peptides to carrier protein was determined by comparing the amino acid composition of the carrier before and after conjugation. In general, ~10 to 15 moieties of peptide per carrier moiety were conjugated by this conventional conjugation technique.

The following peptide thyroglobulin and bovine serum albumin conjugate vaccines were made:

F serotype	Structural Pilin Sequence	Residue Position (R)
F71	PQGQGEVSF	R 5-12
F71	NFKQLQGGAAKKG	R 65-77
F72	PQGQGKVTF	R 5-12
F72	NFKKAAGGGGAKT	R 65-75
F9	QGSGQVNFKG	R 4-12
F9	NFKKAATPGGAAKT	R 65-75
F11	IPQGQGKVTFNG	R 4-15
F12	IPEGQGKVT	R 2-12
F1C	NGGTVHFKGEVVN	R5-12
F1	TTVTVNGGTVHF	R4-15

The intravesicular BALB/c experimental model of pyelonephritis and cystitis, as originally described by O'Hanley, was employed to evaluate the protective capacity of structural pilin synthetic peptide conjugate vaccines to prevent subsequent renal and/or bladder colonization by homologous piliated strains at 48 hours after bacterial bladder inoculation. Cohorts of 20 mice per group were vaccinated via intramuscular administration on two occasions with the experimental thyroglobulin and bovine serum albumin conjugate pilin A vaccines (i.e., day 0 and day 14). Each intramuscular dose of experimental vaccine consisted of ~200 ug in 100 ul saline emulsified in 100 ul incomplete Freund's adjuvant. Control vaccines consisted of thyroglobulin and bovine serum albumin emulsified in saline and incomplete Freund's adjuvant. These control vaccines were administered in similar fashion and schedule as the experimental vaccines. Challenge for pilin A vaccinated mice entailed bladder inoculation of 10^8 CFU of an *Escherichia coli* strain that expressed homologous pili. In contrast, the cohort size for control animals was 5 per challenge strain. The table below summarizes the challenge strategy.

F pilin A vaccine status	Wild-type challenge strain (F serotype expression)
F71	KD201B strain (HB101 transformed with pDA201B) expressing recombinant F71 pili
F72	KD210 B strain (HB101 transformed with pDAL210B) expressing recombinant F72 pili
F9	3669 pyelonephritis strain (F9 pili)
F11	J96 strain expressing F13, plus mannose binding pili
F12	C1979 pyelonephritis strain (F12)
F1C	P2 (F1C)
F1	J198 expresses only mannose binding pili and no Gal-Gal or X binding pili

Protection against subsequent renal colonization and bladder colonization by the challenge strain was defined by >90% of the experimental animals in a cohort (i.e., 18 of 20 vaccinated experimental mice) having no bacterial growth from aliquots of homogenized whole right kidney and aliquots of homogenized whole bladders and none of the experimental animals having heavy bacterial growth from the challenge strain in renal or bladder specimens (viz., the absence of ++++ colonization density or >500 CFU of the challenge strain per 0.1 gram of renal or bladder tissue) compared to heavy growth of

the challenge strain in both renal and bladder specimens (++++ colonization density or >500 CFU per 0.1 gm of renal or bladder tissue) by the challenge strain in >80% of control animals (i.e. ≥4 of 5 control mice).

Results indicated the following:

F serotype	Pilin A Sequence	Residue Positions	Homologous Protection
F71	PQGQGEVT	R 5-12	Yes
F71	PQGQGEVA	R 5-12	Yes
F71	NFKQLQGGAAKKG	R 65-77	Yes
F72	PQGQGKVT	R 5-12	Yes
F72	NFKKAAGGGGAKT	R 65-77	Yes
F9	TTVNGGTVH	R 4-12	Yes
F9	NFKKAATPGGAAKT	R 65-75	Yes
F11	IPQGQGKVTFNGTV	R 4-17	Yes
F12	IPEGQGKVT	R 4-12	Yes
F1C	NGGTVHFKGEVVN	R 5-15	Yes
F1	TTVTVNGGTVHF	R4-15	Yes

One or a combination of pilin A vaccines comprising one or more of the following amino acid sequences that correspond to published and unpublished F pilin primary sequences would be protective against ascending, non-obstructive *Escherichia coli* urinary tract infections in anatomically normal women and males:

F serotype	Pilin A Sequence	Pilin A Residue Positions	Urinary Tract Protection Potential	New or Old Claim
F71	PQGQGEVT	R 5-12	Pyelonephritis	New
F71	PQGQGEVA	R 5-12	Pyelonephritis	New
F71	NFKQLQGGAAKKG	R 65-77	Pyelonephritis	New
F72	PQGQGKVT	R 5-12	Pyelonephritis	New
F72	NFKKAAGGGGAKT	R 65-77	Pyelonephritis	New
F9	TTVNGGTVH	R 4-12	Pyelonephritis	New
F9	NFKKAATPGGAAKT	R 65-75	Pyelonephritis	New
F11	IPQGQGKVTFNGTV	R 4-17	Pyelonephritis	New
F12	IPEGQGKVT	R 4-12	Pyelonephritis	New
F13	PQGQGKVT	R 5-12	Pyelonephritis	Old
F13	AKFGGMGAKKG	R 65-65	Pyelonephritis	Old
F1C	NGGTVHFKGEVVN	R 5-15	Cystitis	New
F1	TTVTVNGGTVHF	R4-15	Cystitis	New

A particularly useful method to produce such a vaccine employs whole pap pili corresponding to F71, F72, F9, and F13 (since they comprise >90% of the wild-type strains responsible for non-obstructive *Escherichia coli* in anatomically normal woman) and

pili expressing R5-12, R4-12, R4-17, and/or R5-15 by insertion into the immunodominant PapA pilin region (R 65-76) using a genetic cassette with mutated *papA* and *papH* cistrons.

In the following examples, mutagenesis of the *pap*H structural gene, which is responsible for anchoring the globoside-binding pili to the cell surface, is utilized. The *pap*H gene was mutagenized of 4 Gal-Gal pilus recombinants, [pHUR849 (*pap*-5), pDAL201B (*Pap*-21), pDAL210B (*pap*-17), and pDAL200A (*pap*-200A)], which encode for the serotypes F13, $F7_1$, $F7_2$, and F9 (2), respectively. This was accomplished by creating deletions of 237 or 300-bp within the *pap*H gene of each strain. These deletions encode for 79 or 100 amino acids respectively, and leads to a truncated form of the PapH protein which allows for the mutant recombinant piliated strains to secrete newly synthesized pili into the culture medium. Since PapH or its truncated form is not required in the secretion or the assembly of the pilin subunit (3), the growing Pap pilus can be detached because of unstable interaction between PapA and the cell envelope. In addition, the complete nucleotide and deduced amino acid sequences of *pap*H genes in all 4 recombinant strains and their deletion derivatives are disclosed herein.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used for these papH examples are listed in Table 1 (below) and Figure 1. The source of the chromosal DNA for pDAL210B was *E. coli* strain 3669, originally isolated from a woman with acute pyeionephritis (2). The source of the chromosomal DNA for pDAL210B and pDAL210B was *E. coli* strain C1212, originally isolated from a woman with acute cystitis (2). The source of the chromosomal DNA for pHUR849 was isolated from *E. coli* strain J96, originally isolated from a woman with acute pyelonephritis (4). All bacteria were cultured in Luria broth or on Luria-agar plates, containing 40 μg/ml X-gal and 20 mM IPTG. Antibiotics were used at the indicated final concentrations: ampicillin, 100 μg/ml, and tetracycline 34 μg/ml, for the selection of plasmid-containing strains. Bacterial transformation were performed as previously described (5).

Table 1. Plasmids used in this study

Plasmid	Description	Reference or source
E. coli		
HB101	F⁻ Δ(*gpt-pro*)62, *leu* B6, *sup* E44, *ara*- -4, *gal* K2, *lac* Y1 Δ(*mcr* C-*mmr*), *rsp* L20(Str^r), *xyl* -5, *mtl* -1, *rec*A13	Stratagene
XL-1 Blue	recA⁻ (recA1, *lac* ⁻, *end* A1, *gyr* A96, *thi* 1, *hsd* r R7, supE44, *rel* A1, (F' proAB, *lac* I^q, *lac* ZΔM15, Tn10))	Stratagene
SURE	e14⁻ (*mcr* A), Δ(*mcr* CB-*hsd* SMR-*mmr*)171, end A1, *sup* E44, *thi* 1, *gyr* A96, rel A1, *lac*, *rec*B, *recJ*, *sbc*C, *umuC* ::*Tn5* (kan^r), *uvr* C, [F' proAB, *lac* I^q, *lac* ZΔM15, Tn10]	Stratagene
OL844	OL1784 *trp*::mTn10	D. Low
Plasmids		
pDAL200A	pUC8 containing a 9 kb Sau 3a DNA fragment encoding for the *pap*-200A operon DNA sequence (see Fig.1)	(2)
pDAL210B	pUC8 containing a 11 kb Eco RI-Sal I DNA fragment encoding for the *pap*-21 operon DNA sequence (see Fig.1)	(2)
pDAL210B	pBR322 containing a 13.5 kb Bam HI DNA fragment encoding for the *pap*-17 operon DNA sequence (see Fig.1)	(2)
pHUR849	pBR322 containing a 11.1 kb Eco RI-Bam HI DNA fragment encoding for the *pap*-5 operon DNA sequence (see Fig.1)	(4)

pKTD-1	pBluescript II containing a 16-bp deletion which removes the Xba I-Sma I multiple cloning site (MCS) of the vector SK- [a]	This study
pKTD-2	pKTD-1 containing a 8-bp deletion which removes the Cla I-Hinc II MCS of the vector SK-	This study
pKTD-3	pBluescript II containing a 8-bp deletion which removes the Eco RI-Hind III MCS of the vector SK-	This study
pKD201B-1	pBluescript II containing a 7.1 kb Eco RI-Kpn I pap-17 DNA fragment derived from pDAL210B	This study
pKD201B-2	pKTD-2 containing a 4.1 kb Hind III DNA fragment derived from pKD201B-1	This study
pKD201B-3	pKD201B-2 containing 3.86 kb Hind III DNA fragment, which contains a 237-bp Cla I-Sma I deletion of *papH*, derived from pKD201B-2	This study
pKD201B-4	pBluescript II containing a 237-bp Cla I-Sma I DNA fragment derived from pKD201B-2	This study
pKD201B-5	pBluescript II containing a 700 bp Eco RI-Cla I DNA fragment derived from pKD201B-2	This study
pKD201B-6	pKD201B-2 containing a 3.2 kb DNA fragment, which contains a 937-bp Eco RI-Sma I deletion	This study
pKD201B-7	pBluescript II containing a 6.86 kb Eco RI-Kpn I DNA fragment, which resulted from the ligation of a 3.86 kb Hind III DNA fragment derived from pKD201B-3, which contains a 237-bp deletion of *papH*, to a 3 kd DNA fragment, derived from pKD201B-1	This study

pKD201B-8	pUC8 containing a 10.86 kb *pap*-21 operon DNA sequence, which resulted from the ligation of a 6.86 kb Eco RI-Kpn I fragment derived from pKD201B-7, which contains a 237-bp deletion of *pap* H, to a 4 kb Eco RI-Sal I fragment, derived from and pDAL210B	This study
pKD200A-1	pKTD-3 containing a 6.4 kb Sal I- Kpn I fragment derived from pDAL200A	This study
pKD200A-2	pKTD-2 containing a 4.1 kb Hind III fragment derived from pKD200A-1	This study
pKD200A-3	pKD200A-2 containing 3.86 kb Hind III DNA fragment, which contains a 237-bp Cla I-Sma I deletion of *papH*	This study
pKD200A-4	pBluescript II containing a 237-bp Cla I-Sma I DNA fragment derived from pKD200A-2	This study
pKD200A-5	pBluescript II containing a 700-bp Eco RI-Cla I DNA fragment derived from pKD200A-2	This study
pKD200A-6	pKD200A-2 containing a 3.16 kb DNA fragment, which contains a 937-bp Eco RI-Sma I deletion	This study
pKD200A-7	pBluescript II containing a 6.16 kb Sal I-Kpn I DNA fragment, which resulted from the ligation of a 3.86 kb Hind III DNA fragment derived from pKD200A-3 to a 2.3 kd Sal I-Kpn I DNA fragment, derived from pKD200A-1	This study
pKD200A-8	pUC8 containing a 8.76 kb *pap* 200A operon DNA sequence, which resulted from the ligation of a 2.6 kb Sal I-Kpn I DNA fragment derived pDAL200A, to a 6.16 kb Sal I-Kpn I DNA fragment, which contains a 237-bp deletion of *papH*	This study

pKD210B-1	pKTD-3 containing 13.5 kb Bam HI DNA fragment encoding for the *pap*-17 operon DNA sequence derived from pDAL210B	This study
pKD210B-2	pKTD-2 containing a 6 kb Hind III DNA fragment derived from pKD210B-1	This study
pKD210B-3	pBluescript II containing a 237-bp Cla I-Sma I DNA fragment derived from pKD210B-2	This study
pKD210B-4	pBluescript II containing a 2.5 kb Eco RI-Cla I DNA fragment derived from pKD210B-2	This study
pKD210B-5	pBluescript II containing a 3.26 kb Cla I-Kpn I DNA fragment derived from pKD210B-2	This study
pKD210B-6	pBluescript II containing a 2.5 kb Eco RI-Sma I fusion-Hind III DNA fragment derived from pKD210B-5	This study
pKD210B-7	pBluescript II containing a 2.5 kb Bam HI-Hind III DNA fragment derived from pKD210B-1	This study
pKD210B-8	pBluescript II containing a 3.3 kb Sma I-Kpn I DNA fragment derived from pKD210B-5	This study
pKD210B-9	pBluescript II containing a 5.77 kb Eco RI-Kpn I DNA fragment resulting from the ligation of a 3.26 kb Sma I-KPN I DNA fragment derived from pKD210B-5, to pKD210B-4 linearized with Cla I-Kpn I, the fusion of Cla I-Sma I creates a 237-bp deletion of *papH*	This study
pKD210B-10	pBluescript II containing a 13.3 kb Bam HI DNA fragment derived from the ligation of a 5.76 kb Hind III DNA fragment derived from pKD210B-9, to a 7.5 kb Hind III DNA fragment derived from pKD210B-1	This study

pKD210B-11	pBR322 containing a 13.3 kb Bam HI DNA fragment *pap* -17 operon DNA sequence derived from pKD210B-10 which contains a 237-bp deletion of *papH*	This study
pKD849-1	pKTD- 2 containing a 4.1 kb Hind III DNA fragment derived from pHUR 849	This study
pKD849-2	pBluescript II containing a 4.1 kb Hind III DNA fragment derived from pHUR 849	This study
pKD849-3	pBluescript II containing a 3.2 kb Sma I DNA fragment derived from pKD849-2	This study
pKD849-4	pBluescript II containing a 3.85 kb DNA fragment derived from the ligation of a 645-bp PCR product derived from pHUR 849, to pKD849-3 linearized with Sma I, which contains a 300-bp deletion of *papH*	This study
pKD849-5	pBR322 containing a 10.8 kb Eco RI-Bam HI DNA fragment encoding for the *pap* -5 operon DNA sequence, which resulted from the ligation pHUR849 linearized with Hind III, to a 3.85 Hind III DNA fragment derived from pKD849-4 which contains a 300-bp deletion of *papH*	This study

[a] MCS of SK- is 657-759 bp, and is flanked by T3 and T7 promoters.

Nucleic acid isolation and manipulations

Large scale plasmid DNA isolation was carried out using a QIAGEN Plasmid Kit (QIAGEN, Inc., Chatsworth, CA). Small scale plasmid mini-preps used for routine DNA analysis were performed using the alkaline lysis method 6). Each protocol provided DNA of sufficient purity to obtain reproducible co-amplification PCR results. The lysozyme boiling miniprep method (7) was used for the isolation of double-stranded DNA templates for sequencing.

Restriction endonucleases, T4 DNA ligase, Kienow fragment of polymerase I, T4 DNA polymerasa, and deoxynucleoside-triphosphates were used according to the conditions recommended by the commercial suppliers (New England BioLabs, Beverly. MA, and Boehringer Mannheim, Indianapolis, IN). After digestion with restriction endonucleases, DNA fragments larger than 1 kb were separated by electrophoresis on 0.7% agarose gels; whereas, separation of smaller DNA fragments was done on 1.5 or 2% agarose gels. Electrophoresis was carried out in TAE buffer (40 mM Tris acetate with 1 mM EDTA [pH 8.0]). DNA fragments were isolated from agarose gels using GENE CLEAN (BIO 101 Inc., La Jolla, CA), or QAlquick Gel Extraction (QIAGEN, Inc., Chatsworth, CA), according to recommendations of the manufacturer.

Oligonucleotide synthesis

Oligonucleotide primers were synthesized by standard phosphoramidite chemistry on a 345 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). After de-blocking at room temperature for 24 h, the primers were recovered by precipitation at room temperature in 1/10 vol of 3 M NaOAc pH 5.2, 2 Vol 100% ETOH. After centrifugation, the pellets were dried under vacuum and resuspended in 200 µl of distilled water. The amount of nucleic acid was estimated by their absorbance at 260nm. All samples were adjusted to the same concentration. Table 2 lists the thirteen different DNA primers used for both sequencing and PCR analysis. PCR amplification was used to determine the orientation of sub-clones containing deletions within the *pap*H gene of each construct.

DNA sequencing and sequence analysis

Double-stranded DNA sequencing was performed using the dideoxynucteotide chain-termination method (8), using [α^{35}S]-thio-dATP (1000 Ci mmol^{-1}, Amersham, Arlington Heights, 1L), and T7 DNA polymerase (Sequenase, U.S. Biochemicals, Cleveland, OH). Sequencing reactions were performed in both directions to confirm the analysis. The oligonucleotide primer 200aRE was used for sequence analysis of the final deletion constructs pKD200A-8 and pKD210B-11. The oligonucleotide PapHRE was used for sequence confirmation of the final deletion constructs pKD201B-8

and pKD849-5. Analysis of DNA and protein sequences used programs distributed through the University of Wisconsin Genetics Computer Group. Nucleotide and Amino Acid sequences were aligned with LINEUP and PRETTY programs (9).

TABLE 2. Primers used in this study

Primers	Oligonucleotide sequence	Description
T3	5' ATTAACCCTCACTAAAG 3'	anneals to multiple cloning site of SK-
T7	5' AATACGACTCACTATAG 3'	anneals to multiple cloning site of SK-
Reverse	5' AACAGCTATGACCATG 3'	anneals to multiple cloning site of SK-
PapHFD	5' ATGAGACTGCGATTCTCTGT 3'	anneals to the TAC translational start region of all 4 *pap* H genes
PapHRE	5' TCCGTTTCTCACAATTCTGA 3'	anneals to bp 509-528 of the *pap* H gene of pDAL201B, *pap*-21 and pHUR 849, *pap*-5
210bFD	5' CCTGAAATACGAGAATATTA 3'	anneals 93-bp upstream of the TAC translational start region of the *pap* A gene of pHUR849, *pap*-5 (2)
210bRE	5' TAATATTCTCGTATTTCAGG 3'	the complement of 210bFD and anneals to the same 93-bp region as described for 210bFD
FOR210b	5' TGGACTGGTATAACAATCGA 3'	anneals 2.9 kb upstream of the TAC translational start region of the *pap* H gene of pDAL210B, *pap*-21
200aRE	5' TCCGTTTCGCACAATTCTGA 3'	anneals to bp 511-528 of the *pap* H gene of pDAL210B, *pap*-17, and pap 200a, respectively
PapFOR[a]	5' AGTGGATTCATGCAGCATTTCT AGAAA 3'	anneals to bp 258-270 of the *pap* A gene of pHUR849, *pap*-5 (2)
FORSEQ	5' TGGACCTCCTGAGCTA 3'	anneals to bp 456-474 of the *pap* A gene of pHUR849, *pap*-5 (2)
PapREV[b]	5' GGGGCAGCCCTGCCGTCCCAA AT 3'	anneals to bp 122-142 of the *pap* H gene of pHUR849, *pap*-5
REVSEQ	5' AAACACCATGAAACACACA 3'	anneals to bp 41-61 of the *pap* H gene of pHUR849

[a] contains a single Bam HI restriction site single underlined.
[b] contains a single Sma I blunt end restriction site double underlined.

DNA amplification

DNA amplification was carried out with 50 ng of plasmid DNA: 0:75 μM of each oligonucleotide in distilled H_2O, supplemented with 1% Triton X-100, 2mM $MgCI_2$, 200 μM each dNTP, and 1.25 U *Taq* DNA Polymerase (Promega, Madison, WI), in a final volume of 100 μl. PCRs were performed in a Epicomp DNA Thermal Cycler (Epicomp, San Diego, CA). All manipulation were carried out with dedicated DNA-free pipettes using Elkay filter pipet tips (Applied Scientific, San Francisco, CA), in a sterile field to minimize the risk of contamination. All reagents were added together except for the *Taq* DNA Polymerase. The reaction mixture was overlaid with 100 μl of sterile mineral oil and was denatured in the thermal cycler at 95°C for 2 min. Then, *Taq* polymerase was added and amplification was earned out over 35 cycles, as follows: a 2 mm denaturation step at 94°C, a 1 min annealing step at 50°C, a 1 min primer extension step at 72°C, and finally, products were extended for 7 mm at 72°C. The reaction mixture was held at room temperature until required. Blank control tubes containing all reagents except the template DNA or primers were also run. The amplified DNA fragments were electrophoresed on 1.5% agarose in TAE butter and visualized by staining with ethidium bromide, and the products were photographed under UV light. PCR was used to establish the correct orientation of the DNA fragments bearing the 237 or 300-bp deletions of the *pap*H gene of each of the four deletion derivatives. The amplification of each construct employed at least one or more different pairs of DNA primers. For each deletion derivative, the DNA primers were as follows: REVERSE and 200aRE for pKD200A-7, REVERSE and PapHRE for pKD201B-7, PapFOR and 200aRE or 210bRE for pKD210B-9, and PapFOR and PapHRE for pKD849-4.

Electron Microscopy

A single colony of each *papH* mutants, their parent recombinants, and the original wild type strains was isolated from a 18 h 37^{0}C growth on agar, suspended in 500 ul of saline, and processed for standard negative staining for transmission electron microscopy. Also, the broth culture of each bacterial strain was processed for negative staining for observation in the electron microscope.

Hemagglutination Assay

Binding properties of strains were determined by slide agglutination using human P1 erythrocyctes as described previously (10). Also, the agglutination of purified pili was performed as described by Normark *et al* (11), using 2-fold serial dilution's starting at 500 μg/ml of protein. A positive reaction was determined macroscopically.

Pili Purification

After 24 h incubation at 37°C in selective broth medium, bacteria were harvested by centrifugation and pili were purified essentially according to the method of Korhonen *et al* (12). The purity of pili preparations was analyzed on SDS-PAGE by silver staining.

Immunoreactivity

The purified pili from each *papH* mutant were assessed for immunoreactivity against polyclonal murine and rabbit antibody reactivity in standard ELISA tests and polyclonal murine and rabbit antibody in Western blotting tests.

Vaccine Efficacy

The efficacy of purified pili from each *papH* mutant was assessed in the standard experimental BALB/c model of pyelonephritis. Cohorts of 20 female mice that were 14 weeks old were immunized intramuscularly on day 0 and day 14 with 50 ug of purified pili from each *papH* mutant, as determined by Lowry technique. Each vaccinal administration consisted of 100ul of pili-incomplete Freund's adjuvant emulsion. Mice were challenged intravesicularly on day 30 by 10^6 bacteria expressing the homologous pili antigen. Challenge strains included: J96 for KD849-5 vaccine recipients; 3669 for KD2001-8 vaccine recipients; KD201 for KD201-8 vaccine recipients; and KD210B for KD210B-11 vaccine recipients. Protection against renal colonization by the challenge strain was assessed at day 2 after challenge. Positive controls included cohorts of 5 non-vaccinated mice challenged with each strain of bacteria. The pili vaccine conferred protection if the right renal homogenates did not reveal any bacterial growth in >90% of the cohort and

none of the renal homogenates in the cohort had more than 5 CFU per gram of tissue. For comparative purposes, all right kidney homogenates from control animals needed to have > 100 CFU of the challenge strain per gram of tissue.

Results

Nucleotide sequences and deduced *Pap*H primary structures

The plasmids pHUR849 (*pap*-5), pDAL201B (*pap*-21), pDAL210B (*pap*-17) and, pDAL200A (*pap*-200A), in *E coli* strain HB101 express digalactose-binding of the serotypes F13, $F7_1$, $F7_2$ and F9, respectively. The *pap* gene cluster responsible for regulation and biogenesis of these pili from *E. coli* strains J96, C1212 and, 3669 is diagrammed in Figure 1. Sequence analysis of *pap*H genes from pDAL201B (*pap*-21), pDAL210B (*pap*-17) and, pDAL200A (*pap*-200A), was compared to the known nucleotide sequence of *pap*H gene of pHUR849 (*pap*-5) (3). Figures 2 shows a single 588-bp open reading frame with the same polarity as *pap*A (2, 4). Analyses of these *pap*H sequences revealed many typical features of prokaryotic gene organization. All four *pap*H gene sequences contained a potential ribosome-binding sites, ATG initiation codon signal sequence, and a TGA termination codon. A potential initiation codon ATG at position -22, preceded by a sequence corresponding to -AGGGT, which showed homology to ribosome-binding sites, was found 13-bp upstream in all four *pap*H sequences. A protein initiated here and ending at the TGA triplet at position 586 would encode a 195 amino acid polypeptide with a calculated molecular weight of 21.9 kd. The mature PapH protein contains 173 amino acid residues. The NH_2-terminal amino acid sequence of the open reading frame has all the features of a signal peptide sequence. The deduced putative signal sequence for the *pap*H was located 22 codons upstream of their terminal Ala (Figure 2). These sequences contained a highly hydrophobic region comprising an amino acids stretch of Ser-Val-Pro-Leu-Phe-Phe-Phe. There was a positively charge amino acid residue (Arg) at the position -21. The suggested cleavage sites between Ala -1 and gly +1 conforms to rules of prokaryotic signal cleavage sites and was similar to most other bacterial genes (12). In addition, the final *pap*H deletion derivatives, pKD849-5 (*pap*-5), pKD201B (*pap*-21), pKD210B-11 (*pap*-17) and pKD200A-8 (*pap*-200A), were also sequenced. In addition,

sequencing into the *pap*A and *pap*C genes which flank the *pap*H gene (Figure 1) of all four *pap*H deletion derivatives was carried out in order to insure that all three genes were in frame. Finally, the codon usage of the *pap*H genes of pDAL201B, pDAL210B and, pDAL200A, and *pap*H gene of pHUR849 were analyzed using a codon frequency computer program (13). The pattern of codon utilization was not significantly different among the genes.

Comparison of papH nucleotide and PapH amino acid sequences

LINEUP and PRETTY computer programs (9) were used to calculate the overall percent homology of the predicted *Pap*H polypeptide at the nucleotide and amino acid level. Figures 3 and 4 compare the deduced nucleotide and amino acid sequences of the *papH* genes of pDAL201B, pDAL210B and, pDAL200A, to the known nucleotide and amino acid sequences of the *pap*H gene of pHUR849. The overall homology among pDAL201B, pDAL210B and, pDAL200A, *pap*H genes was greater than 99% at the nucleotide level and 100% at the amino acid level. Compared to the nucleotide sequence corresponding of *pap*H gene of pHUR849, there was 98% and 99% homology at the nucleotide level and amino acid level, respectively. The amino acid differences among these three *pap*H genes were evaluated and compared to the amino acid sequence of the *pap*H gene for pHUR849 and are shown in Figures 4. Comparisons of the substituted amino acid between pDAL20lB, pDAL210B, pDAL200A and, pHUR849 *pap*H genes shows that there were only two amino acid changes among these genes, and both these changes were non conserved amino acid substitutions. The first non-conserved substitution is Gly→Cys, at amino acid residue -13 of the putative signal sequence, and the second non-conserved substitution Is Val→Ala at amino acid residue 121 of the mature PapH protein. The deduced mature PapH protein of 173 amino acid residues shares many structural features with known *E. coil* pilins. PapH contains two cysteine residues 38 amino acids apart in the NH_2- terminal half of the protein, a tyrosine residue as the penultimate amino acid, and shows an overall sequence similarity to other *E. coli* pilins, especially from Gly 23 to Gly 50 and in the COOH-terminal region (3). These data show that there is genetic

conservation of the *pap*H genes among these strains at both the nucleotide and amino acid level.

PapH deletion mutants

Figure 5 compares the deduced amino acid sequence of the *pap*H deletion mutants, pKD201B-B, pKD210B11, and pKD200A-8, to the amino acid sequence of the deletion mutant of pKD849-5 The final construct pKD849-5 (*pap*-5), contains a 300-bp deletion (nucleotides 145-445, Figure 2), which encodes for 100 amino acids residues (R 27-126). This deletion mutant now contains an open reading frame (ORF) of 219-bp which encodes for a mature fusion protein of 73 amino acids. As shown in Figure 5, the final constructs pKD201B-8 (*pap*-21), pKD210B-11 (*pap*-17) and, pKD200A -8 (*pap*-200A), all contain a 237-bp deletion (nucleotides 207-445, Figure 2), which encodes for 79 amino acids residues (R 48-126), respectively. All three mutants, carry ORF's of 282-bp, which encodes for a mature fusion protein of 94 amino acids. The 94 amino acids are identical among these constructs. In addition, pKD201B-8, pKD210B-11 and pKD200A-8 each contain, one non conserved amino acid substitution at amino acid residue 121 (Val→Ala). Among these strains, this single amino acid substitution within each gene due to a change in the coding sequence of *pap*H gene. This alteration in the coding sequence of the final constructs (Table 1, Figures 2 and 5) arose following the self-ligations of the end-filled Cla 1 to Sma 1 restrictions sites in the intermediate constructs pKD201B-3, pKD210B-9 and pKD200A-3, respectively.

Electron Microscopy

Each of the negative stained *papH* mutants viewed under the transmission electron microscope revealed essentially few if any protruding pili-like structures from the cell surface. In contrast, negative stained bacteria from the parent recombinants and their original wild type strains reveal great numbers of pili-like structure protruding from the cell surface under similar condition of preparation. Also, broth cultures from each *papH* mutant processed for electron microscopy revealed pili free of cellular debris.

Pap H Mutations Affect Cell Association of Pap Pili

E. coli strain HB101 harboring pHUR849, pDAL201B, pDAL210B, and pDAL200a and their *papH* mutants, pKD849-5, pKD201B-8, pKD210B-11, and PkD200A-8, respectively, were assessed for hemagglutination. A single CFU of each strain grown on agar agglutinated human P1 erythrocytes. Also, purified pili from the recombinant strains with an intact *pap* operon or a *papH* mutant agglutinated human P1 erythrocytes. The culture supernatants of *papH* mutants were sufficient to hemagglutinate P1 erythrocytes; whereas, the parenteral recombinants would only hemaggluttinate erythrocytes when pelleted cells were used. These results suggest that *papH* mutants secrete functional Gal-Gal pili into culture supernatant. prepared from these strains.

Pili Isolation and Pili Characterization from *papH* Mutants

The pili from each *papH* mutant purified by a standard method (10) revealed a single band in SDS-PAGE corresponding to the putative PapA moiety of the respective PapA of the parent recombinant and their original wild type strain.

The amount of purified pili from each *papH* mutant strain obtained from 1 liter 18 h broth culture was estimated to be >10 mg. This was calculated by taking the average of 3 protein determinations of aliquots of known diluted purified pili preparation. A Lowry technique was used to estimate protein concentration.

Each purified pili preparation from *papH* mutants was bound by homologous murine and rabbit antisera raised against whole pili of the respective parent recombinant and their original wild type strain in ELISA tests. Identical binding patterns and kinetics were observed among the pili preparations from *papH* mutants, recombinant pili, and wild type pili with these antisera. Also, identical immunoreactivity of the purified pili from *papH* mutants was demonstrated by Western blots with murine and rabbit antisera elicited against whole pili of the respective parent recombinant and their original wild type.

In addition, cohorts of mice immunized with purified pili from each *papH* mutant were protected from subsequent renal colonization./infectivity by the challenge strain. None of the control mice were protected from renal colonization/infectivity by the challenge strain.

Summary

The inventors have mutagenized the *papH* gene of 4 Gal-Gal pilus recombinants, pHUR849 (*pap-5*), pDAL201B (*pap-21*), pDAL210B (*pap17*), and pDAL200A (*pap-200A*). These recombinants encode for the serotypes F13, F71, F72, and F9 (2) respectively. They are intended to be used in large-scale pili vaccine production. This was accomplished by cloning the *papH* gene of these recombinant strains to determine the nucleotide and deduced amino acid sequence of these genes. When compared to the previously published nucleotide and amino acid sequence of the *papH* gene of pHUR849 (3), the recombinant stains pDAL201B, pDAL210B, and pDAL200A were 98% and 99% homologous at the nucleotide level and amino acid level respectively. Based on these sequences, employing PCR and standard recombinant DNA techniques, it was possible to create specific deletions within each *papH* gene. This resulted in 4 recombinant deletion derivatives of pHUR849, pDAL201B, pDAL210B, and pDAL200A, known as pHUR949-5, pDAL201B-8, pDAL210B-11, and pDAL2—A-8. The recombinantpKD849-5, contains a 330-bp deletion that encodes for 100 amino acid residues. This deletion derivative contains an open reading frame of 219-bp that encodes for a mature fusion protein of 73 amino acids. The final constructs pKD201B-8, pKD210B-11, and pKD200A-8 carry 237-bp deletion that encodes for an identical mature fusion protein of 94 amino acids. In addition, we also sequenced into the *papA* and *papC* genes that flank the *papH* gene of all four deletion derivatives to insure that all three genes were in frame for each recombinant. This extended sequence analysis was vital for two reasons. First, the *pap*A , *pap*H, and *pap*C genes are coregulated at the transcription level. The transcriptional activity of these genes is dependent on the transcriptional activity of *pap*I and *pap*B genes (3,14-17). Second, mutations in both *pap*A and *pap*C completely abolish pilus formation and the expression of the adhesion on the cell surface; whereas mutations in *pap*C alone do not affect the pilin antigen produced within the cells (18).

Broth culture results using the *E.* coli bacterial strain HB101 containing these deletion derivatives show that these constructs now release newly synthesized pili fibers into the culture medium. Moreover, these results are consistent with other studies on

the regulation and biogenesis of Pap pili (1, 3, 11, 14-20). In studies, carried out by Nomark and co-workers (3, 18), they have shown that in two different mutations in the *pap*H gene in one strain that 50%-70% of the total pilus antigen was found free of the cells in the culture supernatant in the form of a polymerized structure. The results of the present invention indicate that high amounts of newly synthesized pili fibers are released into the supernatant by the present *papH* mutants. It is also important to note that these pili from the *papH* mutants have identical PapA pilin molecular weights as their parent recombinant and wild type strain. Furthermore, they are immunologically similar to the parent recombinant pili and wild type pili by allowing specific antibody binding to occur in ELISA tests and Western blots. From a vaccine production perspective, these pili can be readily isolated and purified and retain their protective vaccine capacity as demonstrated by their eliciting protection against experimental BALB/c pyelonephritis. Since our data confirm the highly conserved nature of the anchoring gene of Gal-Gal binding piliated bacteria, this strategy of mutagenizing the anchoring gene of the pilus by deletions or other means will be used for other Gal-Gal binding pili belonging to classical F8, F10, F11, F12 serotypes and variant piliated strains (F1C) and uncharacterized pili types in order to collect dissociated pili in broth culture.

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The invention has been described above with reference to specific examples. Further modifications and variations known to those of ordinary skill based on the description herein are contemplated to be within the invention.

The disclosures of all cited references are expressly incorporated herein to the same extent as if each was individually incorporated by reference.